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Spatial organization of the extracellular matrix modulates the expression of PDGF-receptor subunits in mesangial cells

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Spatial organization of the extracellular matrix modulates the expression of PDGF-receptor subunits in mesangial cells. The aim of this study was to test the hypothesis that changes in the extracellular matrix environment regulate rat mesangial cell growth by modulation of the expression of both PDGF-receptor α- and β-subunits. We investigated the mitogenic effects of the PDGF isoforms AA, AB and BB in conventional two-dimensional (2D) culture on laminin, fibronectin, type I, IV and V collagen and in the different spatial organization of matrix in type I collagen gels in three-dimensional culture (3D). In 2D culture PDGF BB was a potent mitogen, AB elicited an intermediate response while AA had no effect on cell proliferation. Extracellular matrix did not modify the PDGF responsiveness in 2D-culture. The different effects of the three PDGF isoforms were due to differential expression and isoform specific association of the PDGF-receptor subunits. Specifically, the β-receptor was strongly expressed, whereas the α-receptor was only barely detectable on the cell surface. Metabolic labeling revealed synthesis and intracellular accumulation of the complete α-receptor protein, and treatment with suramin increased its surface expression, suggesting continuous receptor down-regulation by endogenous PDGF. Morphological and ultrastructural analysis in 3D culture revealed a change in mesangial cell phenotype, forming a branching network of multicellular structures. Assessment of proliferation in 3D culture showed quiescent cells and PDGF unresponsiveness. Investigation of the PDGF β-receptors revealed a rapid down-regulation in 3D culture; both receptor subunits were not detectable on the cell surface. We conclude that 3D culture promotes the induction of a different mesangial cell phenotype. The lack of a mitogenic response to PDGF in 3D culture is due to the down-regulation of the PDGF β-receptors driven by the surrounding matrix. The nonproliferating but metabolically active cells in 3D culture appear to be more closely related to the *in vivo* phenotype than mesangial cells in conventional 2D culture.

Glomerular mesangial cells *in vivo* form a contractile, metabolically active, specialized pericapillary tissue with low mitotic activity. Due to its unique location branching in a tree-like organization between the glomerular capillary loops and to the permanent contact with soluble factors through the fenestrated glomerular endothelium, the mesangium is exposed to a variety of potential stimuli and is a primary target for the action of growth factors such as PDGF [1]. Mesangial cell proliferation and/or accumulation of extracellular matrix components is the uniform response of the renal glomeruli to a variety of inflammatory or other pathogenic agents and is a frequent finding in almost all types of progressive glomerular diseases [2]. This process is the common pathway which may progress to end-stage renal failure and uremia [3].

Previous work has shown that the mesangial matrix is a dynamic compartment. Normal glomeruli contain collagen type IV, type V, laminin, fibronectin and glycosaminoglycans [4, 5]. In disease, profound changes in the quantity and composition of the extracellular matrix have been observed [6–9]. The increased deposition of interstitial collagens type I and III may facilitate platelet adhesion [10, 11] and the release of inflammatory and mitogenic platelet factors. Mesangial cells *in vitro* have been shown to synthesize and secrete matrix components including collagens type I, III, IV, V, laminin, fibronectin and proteoglycans [12–17] and may contribute substantially to the accumulation of glomerular extracellular matrix in progressive disease. However, the mechanisms of mesangial proliferation and matrix accumulation are still incompletely understood, and changes in extracellular matrix composition and spatial organization may influence the mitotic and metabolic activity of mesangial cells. It has been shown that mesangial cells express the β_1 and β_3 [18–20] integrin-receptors for extracellular matrix components, and these receptors likely endow mesangial cells with the ability to respond differentially to changes in the extracellular environment. We hypothesize that variations in the extracellular matrix composition and organization may modulate the responsiveness of mesangial cells to growth factors, in part by regulating the expression of growth factor receptors.

To test our hypothesis we have investigated the effects of the three platelet-derived growth factor (PDGF) isoforms on rat mesangial cells cultured in different extracellular matrix environments. We have chosen PDGF for the following reasons: (1) PDGF is thought to be a paracrine and autocrine growth factor for mesangial cells. Previous work has demonstrated the secretion of PDGF-like activity in the culture supernates of mesangial cells [21], and subsequently the presence of the mRNAs for the PDGF A- and B-chain in mesangial cells has been reported [22]. The expression of the mRNAs for both PDGF chains has been shown to be regulated by various proinflammatory growth factors, thrombin, phospholipids and endothelin [23–26]. (2) PDGF has been shown to be not only a mitogen for mesangial cells [23], but to induce mesangial cell contraction, an increase in the cytosolic calcium and phospholipase activity [27, 28], and

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to modulate prostanoid production in mesangial cells [29]. These effects of PDGF strongly suggest the presence of PDGF receptors in mesangial cells.

However, the presence of the PDGF receptor α- and β-subunit and isoform-specific effects of PDGF AA, AB and BB in mesangial cells cultured in different ECM environments have not been previously investigated. We report here that isoform-specific PDGF effects on mesangial cells are due to a differential expression and isoform-specific association of the PDGF receptor α- and β-subunits. We also show that the spatial organization of the extracellular matrix in three-dimensional culture modifies the effects PDGF isoforms by regulation of the expression of its receptors. In addition, our data demonstrate the induction of a distinct mesangial cell phenotype and of a particular inter-cellular organization in the three-dimensional culture environment.

Methods

Cells

Rat mesangial cells were obtained by previously described methods [14, 30], subcultured weekly at a split ratio of 1:4 by incubation with PBS/5 mM EDTA (Sigma Chemical, St. Louis, Missouri, USA) for five minutes and subsequent incubation in 0.01% Trypsin-EDTA. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, New York, USA) with 10% heat-inactivated fetal calf serum (FCS, Gibco), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, 20 mM Hepes, 1 mM pyruvate (all from Sigma), insulin, transferrin and selenium (ITS Premix, Collaborative Research, Bedford, Massachusetts, USA). Cells between the 4th and 10th passages were used for the experiments.

Matrix protein substrates for two- and three-dimensional cell culture

Human plasma fibronectin (Fn), murine Engelbreth-Holm-Swarm (EHS) tumor-derived laminin (Ln), calf dermal type I collagen (I), EHS-derived type IV collagen (IV) and human placenta-derived type V collagen (V) were prepared and purified as described [4, 5, 31–33].

For experiments in two-dimensional culture bacteriologic plastic dishes (Falcon Labware, Oxnard, California, USA) were coated overnight at 4°C with matrix proteins dissolved in carbonate buffer (pH 9.6). Protein-coating concentrations of 25 μg/ml (Ln) and 12.5 μg/ml (Fn, I, IV and V) have been previously shown to be saturating [34].

In order to study mesangial cells in a three-dimensional environment, gels composed of type I collagen were used [35]. Briefly, type I collagen was solubilized overnight at 4°C in 10 mM acetic acid to achieve a final type I concentration of 5 mg/ml. Collagen was mixed with 10× Earle's salt (10:1) and neutralized with sterile 1 N NaOH. Immediately, mesangial cells were added to achieve a concentration of 10^6 cells/ml collagen. Five hundred microliters of the cell-collagen mixture were transferred to 12 mm Millicell HA tissue culture inserts (Millipore, Bedford, Massachusetts, USA) and placed in 24-well cluster tissue-culture dishes (Costar, Cambridge, Massachusetts, USA). The dishes were incubated for 15 minutes in a 37°C humidified 8% CO_2 incubator to allow polymerization of the gels. Afterwards 1.5 ml of culture medium were added to each well. The equilibration time for macromolecules between two medium compartments separated by the collagen gel was determined to be four hours using horseradish peroxidase type IV as an enzymatic marker [35]. In order to expose mesangial cells in 3D-culture to physiological basement membrane components, 3D gels composed of laminin (0.5 mg/ml) and type I collagen (3.5 mg/ml) were made according to the protocol described above. The homogenous distribution of laminin in the gels was confirmed by immunofluorescence analysis (data not shown). For morphological examination of 3D-cultures the gels were washed three times with PBS, removed from the Millicell tissue culture inserts and snap frozen in OCT embedding compound (Miles Scientific Co., Kankakee, Illinois, USA). Six micrometer cryostat sections of the gels were placed on albumin-coated glass slides, acetone fixed for one min at −20°C and air dried. The sections were examined directly by Hoffman interference or phase-contrast microscopy (Olympus IMT microscope; Ilford XP-1 film) or after Hematoxylin-Eosin staining (Zeiss 14 microscope; Kodak Ektachrome 400 film). Immunofluorescence analyses of the tissue sections were performed as previously described [36] using affinity-purified antibodies to human placental membrane type V collagen and to murine Engelbreth-Holm-Swarm laminin and type IV collagen. Specificity and inter-species cross-reactivity profiles of the antibodies have been previously determined [4, 31]. Samples were examined using a Zeiss Axiophot fluorescence microscope. Photomicrographs were taken at a constant standardized exposure time using Ektachrome 400 film.

Growth factors and antibodies

Human recombinant PDGF AA, AB and BB was purchased from Upstate Biotechnology (Lake Placid, New York, USA). Polyclonal rabbit antisera to a PDGF β-receptor cytoplasmic domain 19-amino acid synthetic peptide (YMAPYDNYVPSAPGRTYRA) and to a β_3 integrin cytoplasmic domain 26-amino acid synthetic peptide (CKWDTANNPIYKEATSTFTNITYRGT) were prepared by immunizing and boosting rabbits using standard methods. The specificity and use of the PDGF β-receptor antibody have been previously described [37, 38]. The rabbit polyclonal antibodies to a cytoplasmic domain of the PDGF α-receptor subunit were a gift of Dr. J.A. Escobedo, University of California, San Francisco (California, USA). The rabbit polyclonal antibody to a β_1 integrin cytoplasmic domain 10-amino acid synthetic peptide (WDTGENPIYK) was a gift of Dr. C. Buck (Wistar Institute, Philadelphia, Pennsylvania). The rabbit polyclonal antibody to the cytoskeletal protein 4.1 was a gift of Dr. W. Horne (Yale University, New Haven, Connecticut, USA).

Proliferation assays

To assess the cell proliferation in two-dimensional culture, tissue culture or matrix-coated 35 mm dishes were washed with PBS before the addition of the cell suspension (2×10^3 cells/cm^2). After 24 hours the regular growth media was replaced by serum free media supplemented with ITS for 48 hours to render the cells quiescent. Subsequently, fresh serum free media supplemented with the appropriate concentrations of the PDGF isoforms were added. In some experiments, the cells were pretreated with 200 μM suramin (Mobay Chemical Co.,

New York, NY, USA) for two hours at 37°C prior to the addition of PDGF. The cell number was determined after four additional days of culture. The cells were detached from the dishes using trypsin/EDTA and the cell number counted in quadruplicate dishes for each condition using an automatic cell counter (Coulter Electronics, Hialeah, Florida, USA). For the determination of mitogenic effects in three-dimensional culture the collagen gels were digested with 1 mg/ml collagenase type II (Sigma Chemical) in PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at 37°C for 60 minutes. This procedure yielded intact, viable cells (>90%) which were counted using a Coulter Counter as described above.

Protein extraction from mesangial cell lysates

Mesangial cell cultures were chilled on ice and washed three times with cold wash buffer (PBS supplemented with protease inhibitors: 2 mM PMSF and 0.16 TIU/ml aprotinin (TIU = trypsin inhibitor unit) (Sigma Chemical). The cells were removed from the petri dish using a teflon scraper (Costar) and suspended in wash buffer. The cells were centrifuged at 1200 rpm for five minutes and the pellet resuspended in lysis buffer (20 mM Hepes, 1% Triton X-100, 20% glycerol, 2 mM PMSF, 0.16 TIU/ml aprotinin). In some experiments additional protease inhibitors were used: 2 mM EGTA, 1 mM DFP and 1 mM leupeptin (all from Sigma Chemical). Approximately 0.5 to 1 × 10^6 cells were lysed in 100 μl lysis buffer. The resulting solution was spun (12000 rpm for 10 min at 4°C) and the supernatant was decanted and stored at −70°C.

To extract proteins from cells in three-dimensional culture, the collagen gels were washed twice with PBS and three times with wash buffer, placed in a 1.5 ml Eppendorf tube in 100 μl lysis buffer and homogenized using a plastic pestle (Kontes, Vineland, New Jersey, USA). The cell and collagen fragments were pelleted and the resulting supernatant was decanted and stored as described above. In parallel experiments the collagen gels were digested with 1 mg/ml collagenase type II, the cells pelleted and lysed with 100 μl lysis buffer.

For the detection of tryptic fragments of the PDGF β-receptor [39], the cells or collagen gels were washed with PBS and serum-free media and subsequently treated with 0.1 mg/ml trypsin (Sigma) in serum-free media for 60 minutes at 37°C prior to protein extraction.

The protein concentration in each lysate was determined spectrophotometrically (BCA Protein Assay Reagent, Pierce, Rockford, Illinois, USA).

Immunoblot analysis

The extracted proteins from mesangial cell lysates were solubilized in 5× SDS loading buffer for five minutes at 100°C and separated by electrophoresis on 6% SDS-polyacrylamide gels [40] under reducing conditions. Subsequently the proteins were transferred to a 0.45 μm-pore nitrocellulose membranes (Schleicher & Schuell, Keene, New Hampshire, USA) by electrophoresis (Polyblot, American Bionuclear, Emeryville, California, USA). Molecular weight markers and total protein were detected by staining with Amido black. The nitrocellulose membranes were blocked (PBS/4% BSA with 0.05% NaN_3) overnight at 4°C. The membranes were washed three times in TBS-T (0.05% Tween 20 in 50 mM Tris, 150 mM NaCl, 0.05% NaN_3, pH 7.4) and incubated with rabbit polyclonal antisera diluted 1:1000 in PBS/4% BSA for two hours at room temperature with gentle agitation. Thereafter the nitrocellulose was washed three times with TBS-T and incubated for one hour at room temperature with an alkaline phosphatase conjugated goat anti-rabbit IgG (Fc specific) antibody (Promega Biotec, Madison, Wisconsin, USA) diluted 1:7500 in TBS-T. After three final washes the nitrocellulose was incubated in AP buffer (10 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5). Secondary antibody bound to the nitrocellulose was detected by incubation with a substrate solution (Sigma), which consisted of nitroblue tetrazolium (330 μg/ml) and 5-bromo-chloro-3-indolyl phosphate (165 μg/ml). The color development was stopped after 5 to 15 minutes by washing the nitrocellulose with 20 mM Tris, 5 mM EDTA, pH 8.0.

Immunoprecipitation

Cells for surface labeling in two-dimensional culture were grown on the different ECM components for one or seven days. In some experiments the cells were pretreated with 200 μM suramin for two hours at 37°C prior to surface labeling. To allow for surface labeling of mesangial cells in three dimensional culture, the cells were dispersed in acid soluble type I collagen as described above, placed in 50 μl collagen drops in 60 mm-diameter bacteriologic petri dishes and cultured for up to 12 days. Each petri dish contained 20 drops with 5 × 10^4 cells each. This procedure allowed rapid access of the iodination reagents to the cell surface and yielded reproducible iodination results.

The cells were washed three times with PBS and treated with 1 mCi/ml $Na^{125}I$ (Amersham, Arlington Heights, Illinois, and New England Nuclear, Wilmington, Delaware, USA) in PBS supplemented with 100 U/ml lactoperoxidase (Sigma) and 0.005% hydrogen peroxide. The reaction was quenched after 10 minutes with repeated washes with excess cold NaI (20 mg/ml in PBS). For metabolic labeling the cultures were incubated for two hours in serum-free and methionine-free DME (Gibco), washed and further incubated for 18 hours in serum-free and methionine-free DME supplemented with 100 μCi/ml ^{35}S-methionine (Amersham). Cell lysates were prepared as described above and supplemented with 15 μl 2 M NaCl, 10 μl PBS/4% BSA and 5 μl distilled water per 100 μl lysate. The lysates were preabsorbed with 20 μl Sepharose CL-4B (Pierce) with agitation for 60 minutes at 4°C. Lysates labeled with ^{35}S were further preabsorbed with rabbit IgG coupled to Sepharose CL-4B for 60 minutes. The different rabbit polyclonal antisera were diluted 1:25 in PBS/4% BSA, 50 μl added to the preabsorbed lysate and incubated for 90 minutes at 4°C with agitation. The antigen-antibody complexes were precipitated with 20 μl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 90 minutes with gentle agitation. The antigen-antibody complexes bound to the Sepharose beads were pelleted in an Eppendorf microfuge and washed five times with 750 μl of 50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.5. The proteins were solubilized by boiling the Sepharose beads for five minutes in 80 μl of 1× SDS loading buffer and then electrophoresed on 6% SDS-PAGE under reducing (PDGF receptors) or non-reducing (integrins) conditions. Gels were dried, those labeled with ^{35}S after being treated with EN^{3}HANCE (New England Nuclear), and exposed on XAR-5 film (Eastman Kodak, Rochester, New York, USA) at −70°C.

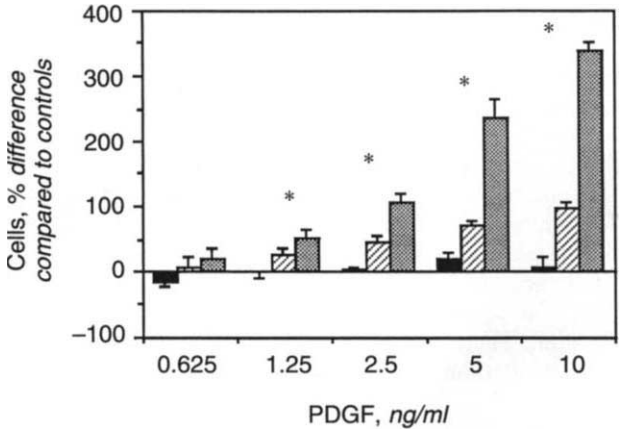

Fig. 1. *Mitogenic effects of PDGF AA (■), AB (▨), and BB (▩) in mesangial cells.* 2×10^5 cells were plated on collagen I coated dishes, made quiescent for 48 hours in serum free-media and stimulated with the three PDGF isoforms in the indicated concentrations in serum-free media for 4 days. The cell number was determined using an automated cell counter and compared to the control experiment without PDGF. The mean cell density in the control dishes was 4.1×10^3 cells/cm^2. The data are given as mean ± SD of quadruplicate dishes. PDGF AB and BB concentrations which induced a significant increase in the cell number ($P < 0.05$) compared to controls are indicated by an asterisk.

The relative density of specific bands was determined by scanning densitometry (Hoefer Densitometer, Hoefer Scientific Instruments, San Francisco, California, USA).

Electron microscopy

The procedure was performed as described previously [41]. Briefly, three-dimensional cultures were washed extensively with PBS, fixed for two hours with formaldehyde-glutaraldehyde and placed in 0.2 M sodium cacodylate buffer. The samples were postfixed in 1% osmium tetraoxide buffered with 0.2 M s-collidine for one hour at 4°C. After rinsing three times with 0.1 M s-collidine, the samples were stained with uranyl acetate/oxalic acid for one hour at 4°C. Dehydration steps were from 70 to 100% ethanol for 10 to 30 minutes, ending with 100% propylene oxide and embedding in Epon 812. Ultrathin sectioning was done on a LKB III 8800 Ultramicrotome (LKB Produkter AB, Bromma, Sweden). The sections were stained with 2% uranyl acetate/lead citrate and viewed on a Zeiss EM 10B electron microscope (Carl Zeiss GmbH, Oberkochen, Germany).

Statistics

All data are presented as mean ± standard deviation. Differences between means were evaluated by the unpaired Student's *t*-test. In all circumstances $P < 0.05$ was considered significant.

Results

PDGF AB and BB are mitogens for mesangial cells in two-dimensional culture

Treatment of mesangial cells with PDGF AB and BB induced a dose-dependent increase in cell number (Fig. 1). This effect was significant with both PDGF isoforms in a concentration as low as 1.25 ng/ml. PDGF BB was a more effective mitogen compared to equivalent concentrations of PDGF AB. Even high concentrations (10 ng/ml) of the AA homodimer had no effect on mesangial cell proliferation. Figure 1 shows the proliferative response of mesangial cells plated on type I collagen. Similar experiments were performed with cells plated on tissue culture plastic, laminin, fibronectin, type IV and V collagen and showed identical results (data not shown). The results of the proliferation assays are consistent with the concept of isoform-specific association of PDGF receptor subunits [42, 43], specifically, that mesangial cells should express both receptor subunits. Since PDGF AA failed to induce any mitogenic effect despite of tyrosine-phosphorylation of the receptor and activation other signal transducing proteins such as the c-*src* protein-tyrosine-kinase (Marx M, Warren SL, Madri JA, manuscript in preparation) it is likely that activation of the $\alpha\alpha$-receptor homodimer may induce other than mitogenic signals in mesangial cells. The mitogenic effects of PDGF AB and BB suggest that the α- and β-receptor subunits dimerize to form the $\alpha\beta$ and $\beta\beta$ receptor dimers and mitogenic signals are possibly transduced preferentially by the activation of the β-subunit.

The α- and β-receptor subunits are expressed on the cell surface in two-dimensional culture, the α-subunit is rapidly processed and internalized

Mesangial cells in two-dimensional culture were found to express the β-receptor subunit on their cell surface. When used in lysates from surface-labeled cells, the β-receptor antibody precipitated two bands of 170 and 140 kD (Fig. 2, lane 1). The higher molecular weight band represents the mature receptor protein, whereas the molecular basis of the 140 kD surface labeled protein coprecipitating with the PDGF β-receptor is yet to be identified. The abundance of the 140 kD band makes it unlikely that it corresponds to a partially- or non-glycosylated precursor form of the β-receptor [39] also expressed on the cell surface. Even though experiments performed in the presence of different protease inhibitor cocktails yielded identical results, we cannot exclude completely the possibility that the lower molecular weight band represents a proteolysis product. The strong surface expression of the β-receptor subunit was constantly reproducible using different cell preparations at passages 4 to 10 cultured on different extracellular matrices and on tissue culture plastic.

Immunoprecipitation studies from surface labeled cell lysates using the α-receptor antibody showed only a faint and barely detectable 180 kD band (Fig. 2, lane 2). It is unlikely, that these results were due to low specificity of the antibody, since we were able to precipitate the α-receptor easily from surface labeled microvascular endothelial and vascular smooth muscle cells (Marx M, Perlmutter RA, and Madri JA, manuscript in preparation). The mitogenic effect of PDGF AB in mesangial cells suggests the presence of the α-receptor subunit in these cells. To clarify these results, we further investigated the cellular localization of the α-receptor subunit. Immunoprecipitation from metabolically labeled mesangial cells demonstrated considerable amounts of *de novo* synthesized α-receptor subunit (Fig. 3, lane 2). The discrepancy between the low or non-detectable surface expression and remarkable presence of metabolically labeled α-receptor prompted us to hypothesize either a predominantly intracellular localization of the receptor in these cells and culture conditions or a rapid turnover of the

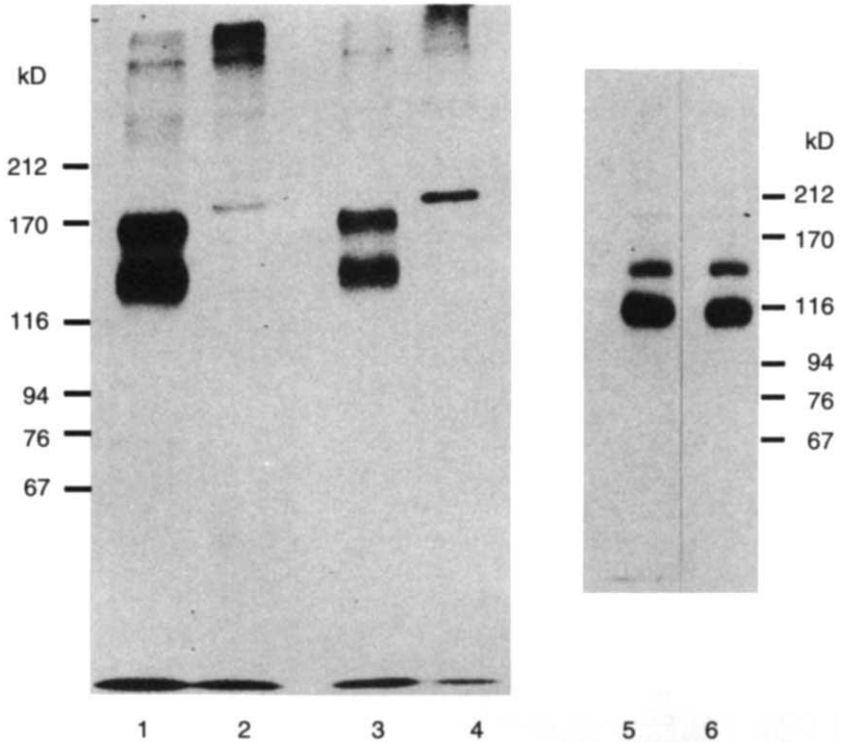

Fig. 2. *Mesangial cells express both PDGF-receptor subunits, the PDGF α-receptor is down-regulated in an autocrine PDGF-loop.* Lysates from ^{125}I surface labeled cells were immunoprecipitated with the β-receptor (lane 1) and α-receptor antibody (lane 2) and analyzed by SDS-PAGE under reducing conditions on 6% gels. The β-receptor antibody recognizes a strong double band (170 and 140 kD). The PDGF α-receptor was only barely detectable as a faint 180 kD band (lane 2). Treatment with suramin (200 μM for 2 hr, 37°C) increased the α-receptor expression on the cell surface 2.7-fold (lane 4) compared to non-treated cells (lane 2). The surface expression of the 170 kD β-receptor decreased moderately (0.72-fold) after suramin treatment (lane 3) compared to non-treated cells (lane 1). The analysis of β_1-integrin (115 kD) surface expression served as an internal control and decreased slightly after suramin treatment (0.8-fold; lane 6) compared to the expression in non-treated cells (lane 5). The 140 kD bands in lanes 5 and 6 represent the co-precipitated integrin α-chains.

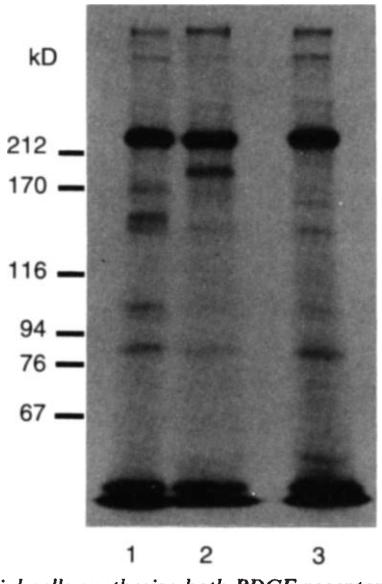

Fig. 3. *Mesangial cells synthesize both PDGF-receptor subunits.* The cells were metabolically labeled with ^{35}S-methionin for 18 hours. The lysate was precipitated with antisera against the PDGF β-receptor (lane 1), α-receptor (lane 2) and with non-immune serum (lane 3) and analyzed on 6% SDS-PAGE under reducing conditions. The β-receptor antibody precipitates a 170 kD (mature receptor) and 140 kD (possible precursor protein) band. The α-receptor antibody recognizes a 180 kD band which represents the mature receptor protein. The strong high molecular weight band (>220 kD) which appears in all lanes corresponds to fibronectin, which is known to be synthesized in considerable amounts in mesangial cells *in vitro* and binds non-specifically to Protein-A Sepharose.

cell surface receptors with quick internalization even in the absence of exogenous PDGF. Since it has been shown that suramin binds to the PDGF receptor and prevents the interaction with its ligand [44, 45], we attempted to interrupt receptor/ligand binding and subsequent receptor internalization of the activated receptor by treatment with suramin. We hypothesized that treatment with suramin should interrupt the suspected rapid internalization process of the α-receptor. Figure 2 (lanes 3 and 4) shows the effect of the exposure of the mesangial cells to 200 μm suramin for two hours: the surface expression of the α-receptor (lane 4) increased dramatically compared to non-treated control cells (lane 2), whereas the expression of the β-receptor subunit (lanes 1 and 3) slightly decreased. The intensity of the specific bands was quantified by scanning densitometry, normalized using the surface expression of β_3 integrins precipitated from the same cell lysates (lanes 5 and 6), and showed a suramin-induced 2.7-fold increase in the α-receptor expression. The intensity of the β-receptor band decreased 0.72-fold. Suramin treatment did not influence the non-responsiveness to PDGF AA in the proliferation assay, whereas the effects of the mitogens PDGF AB and BB were diminished in cells exposed to suramin (Fig. 4).

Three-dimensional culture induces dramatic changes in mesangial cell phenotype and organization

The morphologic and ultrastructural analysis of mesangial cells in three-dimensional type I collagen gels revealed a surprising modification of the cell phenotype and inter-cellular organization. Mesangial cells in conventional two-dimensional

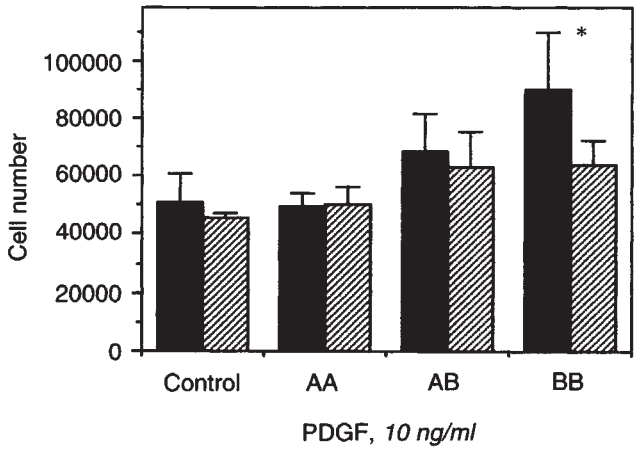

Fig. 4. *Suramin inhibits the mitogenic effects of PDGF BB.* The cells were made quiescent for 48 hours in serum-free media, treated with suramin (200 μM, 2 hr, 37°C) and subsequently exposed to PDGF AA, AB or BB (10 ng/ml) in serum-free media for 48 hours. The cell proliferation was assessed as described in Figure 1. Significant differences ($P < 0.05$) between suramin treated (hatched bars) and control cultures (closed bars) are indicated by an asterisk.

culture grow as elongated, stellate cells, forming bundles and irregular groupings (Fig. 5a). In three-dimensional culture the distinct spatial organization of extracellular matrix induced a dramatic reorganization of the whole cell population. This process was characterized by a marked contraction of the collagen gels, which became visible approximately after 6 to 10 hours. Morphologic analysis of frozen sections of 3D cultures using light microscopy (Fig. 5b) showed a substantial reorganization of the cells forming a complex branching network of multicellular structures which closely resembles the network previously described for vascular smooth muscle cells and a variety of endothelial cells cultured in three-dimensional collagen gels [35, 46]. Using tissue culture inserts with a transparent membrane (Wheaton Scientific, Millville, New Jersey, USA) we were able to analyze and document the cell behavior in 3D culture by photomicrography of living cells. Figure 6a shows the rounded cells immediately after they have been placed in the collagen gels. After 18 hours of 3D-culture the cells already showed impressive elongation and spreading through the gels (Fig. 6b). The multicellular network of mesangial cells was well established after 48 hours of 3D-culture (Fig. 6c). The higher density of cells in Figure 6c is due to the dramatic contraction of the collagen gels and not to cell proliferation. Ultrastructural analysis of mesangial cells in 3D culture (Fig. 7) demonstrated activated cells with abundant endoplasmatic reticulum, increased amount of vacuoles and numerous pseudopodia. The cells abutted one another and formed multicellular aggregates. Occasionally junctional complexes were seen. However, in contrast to capillary endothelial cells [35, 41] no lumen-like structures could be detected. Immunofluorescence microscopy and analysis of extracellular matrix components in 3D cultures revealed the induction of synthesis and deposition of the physiological basement membrane proteins collagen type IV and V (Fig. 8). On day 1 of 3D culture some intracellular stain for type IV collagen was already present (Fig. 8a), whereas staining for type V collagen was completely negative (Fig. 8c). Investigation of 3D cultures on day 4 after the formation of the cellular network disclosed a marked staining for collagen type IV (Fig. 8b) and V (Fig. 8d) along the multicellular structures. These results support the notion, that the mesangial cell phenotype in 3D culture is closely related to the *in vivo* phenotype.

The spatial organization of extracellular matrix in 3D culture prevents the mitogenic effects of PDGF

In 3D culture no mitogenic effects of the three PDGF isoforms even in concentrations as high as 10 ng/ml could be demonstrated. Treatment with medium supplemented with 10% FCS also failed to induce a proliferative response compared to control cultures in serum free medium (Fig. 9).

The loss of mitogenic PDGF effects in 3D culture is due to down-regulation of the PDGF β-receptor subunit

To further investigate the influence of the spatial organization of extracellular matrix on mitogenic effects of the PDGF isoforms, we analyzed the surface expression of both PDGF receptor subunits in 3D culture. Immunoprecipitation from surface labeled cells after nine days of 3D culture failed to reveal the specific bands characteristic for the α- and β-receptor subunit, even after prolonged (60 days) autoradiographic exposure of the gels (Fig. 10a, lanes 1 and 2). This negative result cannot be attributed to reduced cell viability in 3D culture, as ultrastructural analysis, trypan blue exclusion and replating of cells isolated through digestion of the collagen gels demonstrated normal viability. Furthermore, a diminished effectiveness of the surface iodination or protein extraction procedure in 3D culture could be excluded by the strong signal of β_1 and β_3 integrins (Fig. 10a, lanes 3 and 4) precipitated from the same cell lysates as the PDGF-receptors. In addition, no tryptic products [39] of the β-receptor could be detected after nine days of 3D culture (data not shown). To address whether the reduced expression of the β-receptor in 3D culture is due to a reduced synthetic rate or to an accumulation of the receptor protein in the intracellular compartment we investigated the time course of the β-receptor expression in 3D culture by immunoblotting. Equal protein amounts were separated on each lane. Figure 10b demonstrates the presence of the β-receptor protein in 3D culture after 4 and 24 hours, the intensity of the specific bands is similar to the result in 2D culture (Fig. 10b, lane 1). However, during the further time course of 3D culture the amount of the β-receptor protein decreased rapidly and could be only barely detected after nine days of culture. Immunoblotting of the cytoskeletal protein 4.1 revealed that the expression of the 70 kD isoform did not change during the time course of 3D culture and served as an additional control (Fig. 10b, lower panel). Protein extraction from the whole collagen gels or from the pelleted cells after collagenase digestion of the gels yielded identical results in immunoblots. These results were easily reproducible using mesangial cells from different preparations between passages 4 to 10. To investigate the PDGF β-receptor protein in mesangial cells exposed to physiological basement membrane components in 3D cultures, the cells were placed in gels composed of laminin (0.5 mg/ml) and type I collagen (3.5 mg/ml). The addition of laminin made the gel much denser compared to gels composed of type I collagen alone. The gel contraction as marker of metabolic activity was more pronounced compared to type I collagen gels and the cells did not

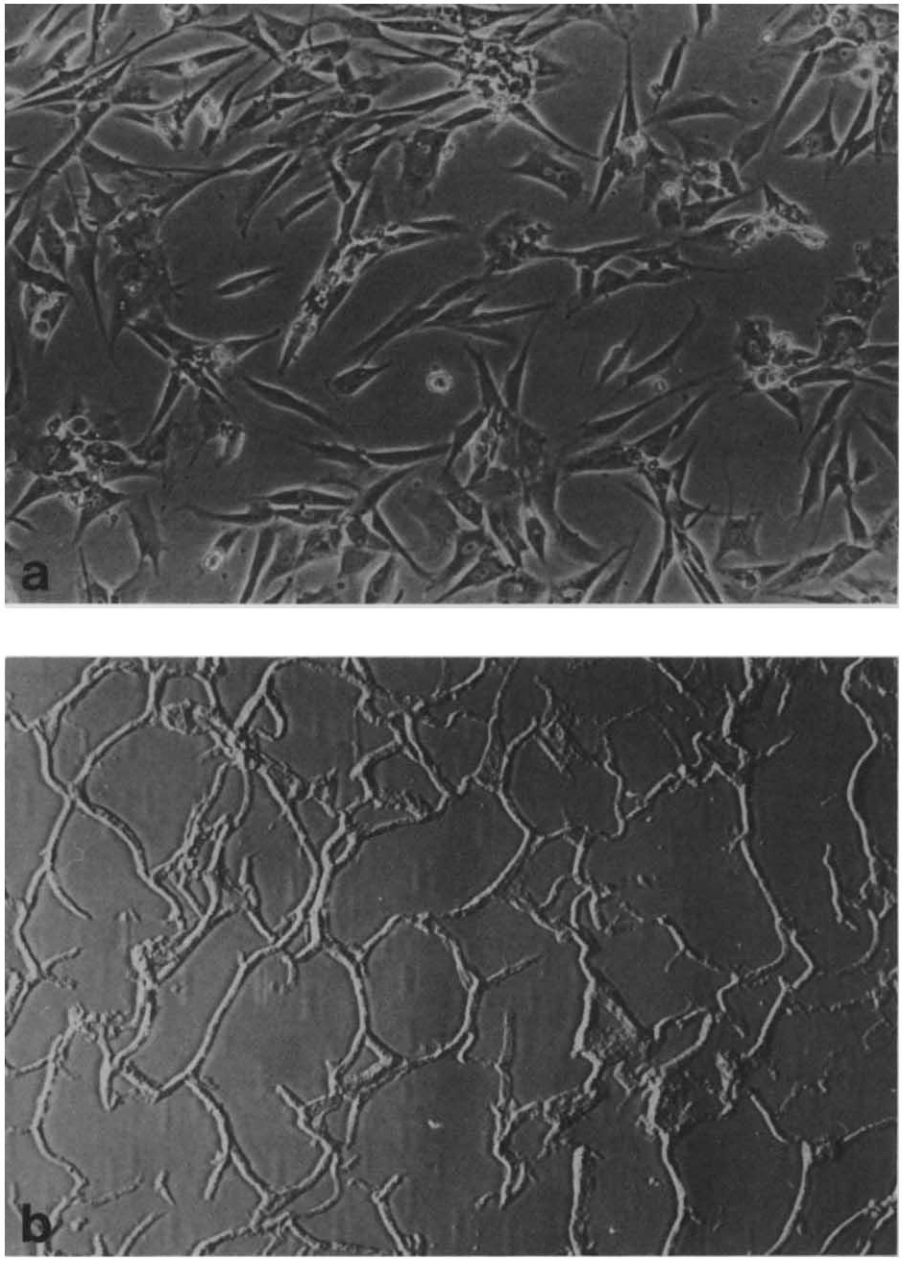

Fig. 5. *Three-dimensional culture induces dramatic changes in cell morphology and organization.* Compared to mesangial cells in conventional two-dimensional culture (**a**, 79×), morphologic analysis in three-dimensional culture (**b**, 79×) showed a branching network of tube-like structures.

proliferate. Analysis of PDGF β-receptor expression by immunoblotting (Fig. 10c) revealed a rapid down-regulation already after one day of 3D culture and on day 3 the β-receptor was only barely detectable. The expression of the cytoskeletal protein 4.1 served as control and remained unchanged during the time course of 3D culture. Since immunoprecipitation from surface labeled cells failed to prove PDGF α-receptor expression on the cell surface in 3D culture, the total cellular protein expression of the α-receptor was also investigated by immunoblotting during the time course of 3D culture. No regulation of the total α-receptor protein could be demonstrated (Fig. 10d).

The surface expression of the β-receptor is not modified by extracellular matrix in 2D culture

To further elucidate a possible influence of different extracellular matrix components on the mitogenic effects of PDGF in 2D culture, the β-receptor subunit was analyzed by western blotting from cell lysates and immunoprecipitation from surface-labeled cells. The surface expression of the β-receptor was not influenced by growing the cells on Ln, Fn, type I, IV and V collagen for seven days (Fig. 11a). In addition no modification of the total amount of the β-receptor protein could be demonstrated in cells cultured on the different matrices (Fig. 11b). Consequently, it was not possible to demonstrate any significant differences in the mitogenic effects of the PDGF isoforms on mesangial cells cultured on Ln, Fn, type I, IV and V collagen (data not shown).

Discussion

Previous work by others strongly suggests a pivotal role of PDGF as a mediator of mesangial cell proliferation in glomerular disease [21–29]. In human renal disease and in experimental

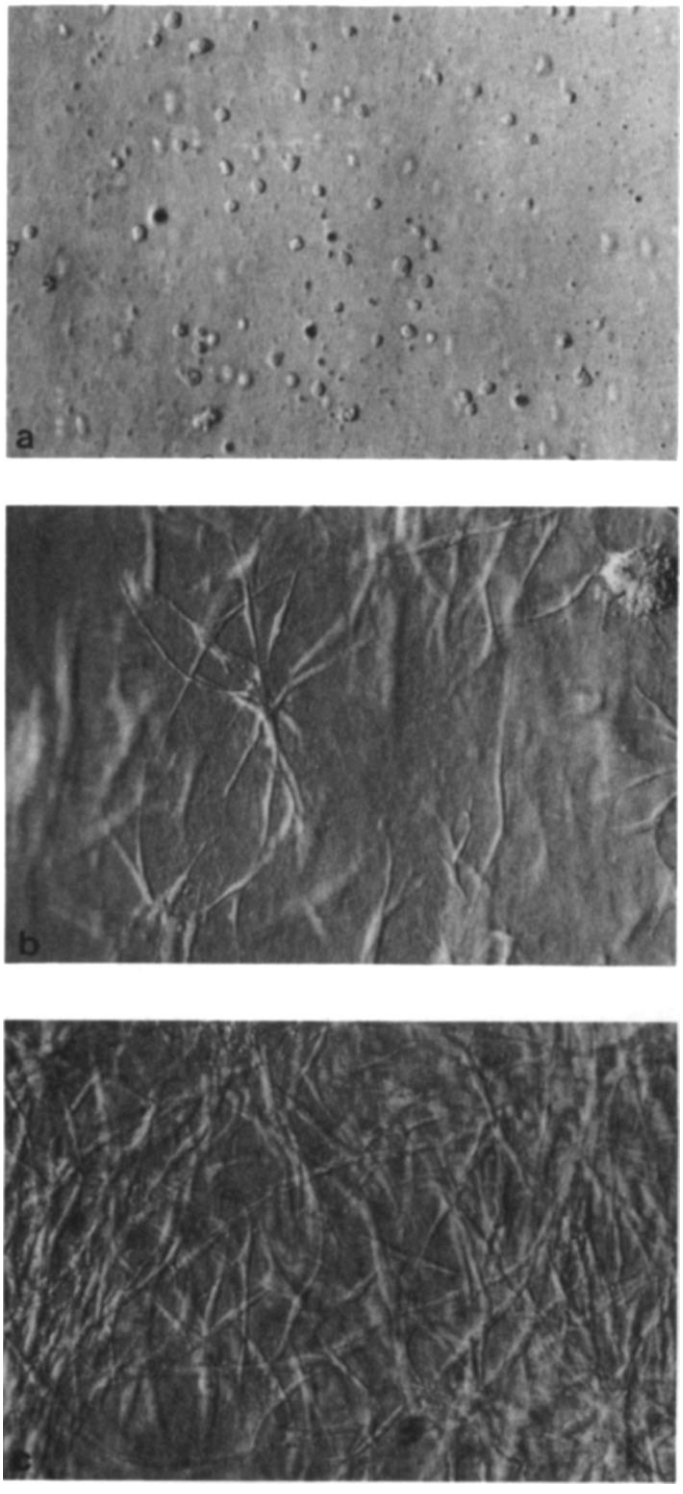

Fig. 6. *Direct observation of living mesangial cells in 3D culture using tissue culture inserts with a transparent membrane.* **a.** (75×) the cells immediately after they have been placed in the gel; **b** (75×) demonstrates the cell spreading after 18 hours of 3D culture and **c** (75×) shows the cellular network after 48 hours of 3D culture. The higher cell density in c is due to the contraction of the gels and not to proliferation.

glomerulonephritis an increase in the PDGF B-chain and PDGF β-receptor expression predominantly in the mesangial region has been reported [47–51]. However, the influence of different extracellular matrix environments on the presence and surface expression of both PDGF receptor subunits and on isoform specific PDGF effects on mesangial cells have not been reported previously.

Our studies have demonstrated marked differences in the action of the three PDGF isoforms on mesangial cells: PDGF AA has no mitogenic effects, AB elicits an intermediate response and BB is a potent mitogen. According to the concept of isoform specific association of both PDGF-receptor subunits [42, 43], binding of PDGF to its receptor requires receptor dimerization and is restricted to ligand-specific association of the receptor subunits: PDGF AA binds only to the αα-receptor homodimer; AB to the αα- and αβ-receptors; and BB to all three possible receptor dimers (αα, αβ, ββ). The different mitogenic effects of the PDGF isoforms in this study indicate that mesangial cells express both PDGF receptor subunits which associate to form the αβ- and ββ-dimers, the reduced mitogenic effect of AB compared to BB can be readily explained by the ability of PDGF BB to bind to all three (αα, αβ and ββ) receptors, thus inducing more tyrosine-kinase activity than PDGF AB whose binding is restricted to the αα and αβ receptor dimers. Alternatively, a reduced ligand binding affinity to the αα- and αβ-receptor is a possible explanation. PDGF AA failed to induce any mitogenic effect.

However, PDGF AA induced receptor autophosphorylation, activation of the c-src protein-tyrosine kinase and changes in cell morphology (Marx M, Warren SL, Madri JA, manuscript in preparation). We conclude from these data that mitogenic signals are preferentially transduced by the activation of the β-receptor subunit, whereas activation of the α-receptor subunit may induce other than mitogenic signals in mesangial cells. Biochemical analysis of the PDGF receptors showed synthesis and strong surface expression of the β-receptor subunit. Very low amounts of the α-receptor could be precipitated from surface labeled cells. However, immunoprecipitation after metabolic labeling showed strong evidence for the synthesis of the complete α-receptor protein in mesangial cells.

The discrepancy between the very low surface expression and remarkable biosynthesis with intracellular accumulation of the complete α-receptor protein prompted us to investigate a possible participation of the α-receptor in a hypothetical autocrine PDGF loop in mesangial cells. The presence of similar autoregulatory mechanisms has been suggested recently in human glioblastoma [52] and smooth muscle cells [53]. In addition, an intracellular activation of PDGF-receptors by autocrine mechanisms has been observed in v-*sis*-transformed rat kidney cells [54]. We were able to induce a rapid increase of the surface expression of the α-receptor subunit by a short (2 hr) treatment with 200 μM suramin, which has been shown previously to bind to the PDGF-receptor and to prevent its internalization [44, 45]. In addition, suramin has been shown to be able to enter cells and to exhibit intracellular effects. In *sis* transformed fibroblasts suramin increased the processing of the PDGF-receptor precursors to the complete receptor protein, suggesting an autocrine intracellular interaction of the *sis* gene product with newly synthesized PDGF receptors [55]. In mesangial cells such an intracellular interaction appears to be of no biological significance as these cells, compared to *sis* transformed fibroblasts synthesize mainly the mature α-receptor protein, since only trace amounts of precursor proteins could be

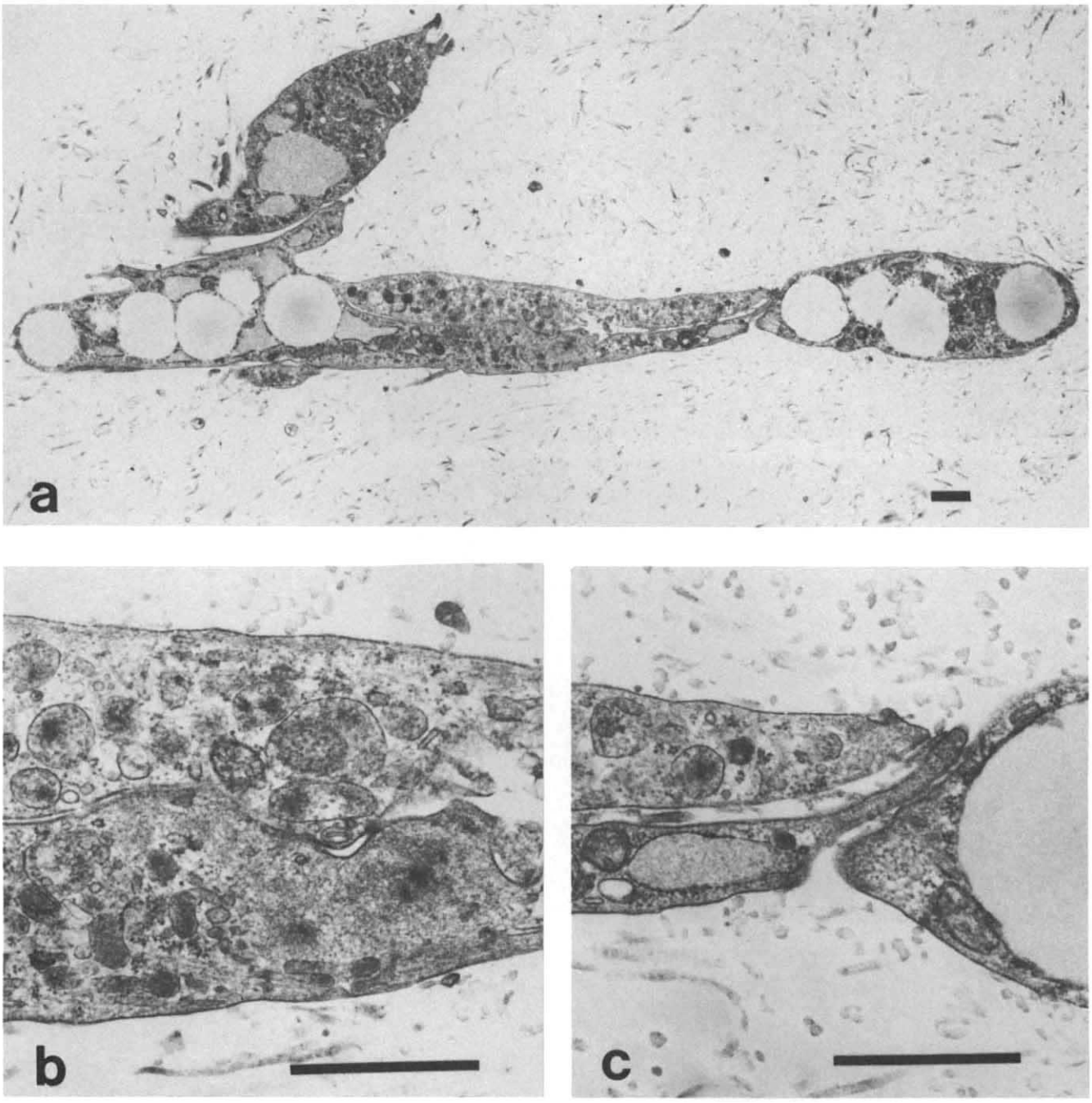

Fig. 7. *Ultrastructural analysis of mesangial cells after 9 days in 3D culture revealed aggregates of activated cells with increased endoplasmatic reticulum, vacuoles and pseudopodia (a, 6.600×).* **b** and **c** show the close cell to cell association and junctional complexes at high magnification (30.000×). The bars represent 1 μm.

detected by metabolic labeling. Taken together with the demonstration of intracellular presence of the mature α-receptor protein our results suggest a rapid down-regulation of the α-receptor with only short presence on the cell surface and a quick internalization even in the absence of exogenous PDGF. In conjunction with the previously demonstrated endogenous production of PDGF by mesangial cells [21–23] this mechanism may well be part of a growth regulatory autocrine PDGF loop. The surface expression of the β-receptor was not increased by suramin, suggesting no noticeable down-regulation of the β-receptor by endogenous PDGF. However, pretreatment with suramin induced an inhibition of the mitogenic effects of exogenous PDGF BB. This effect is compatible with the notion, that suramin indeed interferes with receptor/ligand binding and prevents receptor internalization.

Changes in the amount, composition and organization of the ECM environment are a common feature in a broad spectrum of glomerular diseases [2, 3]. Previous work in our laboratories

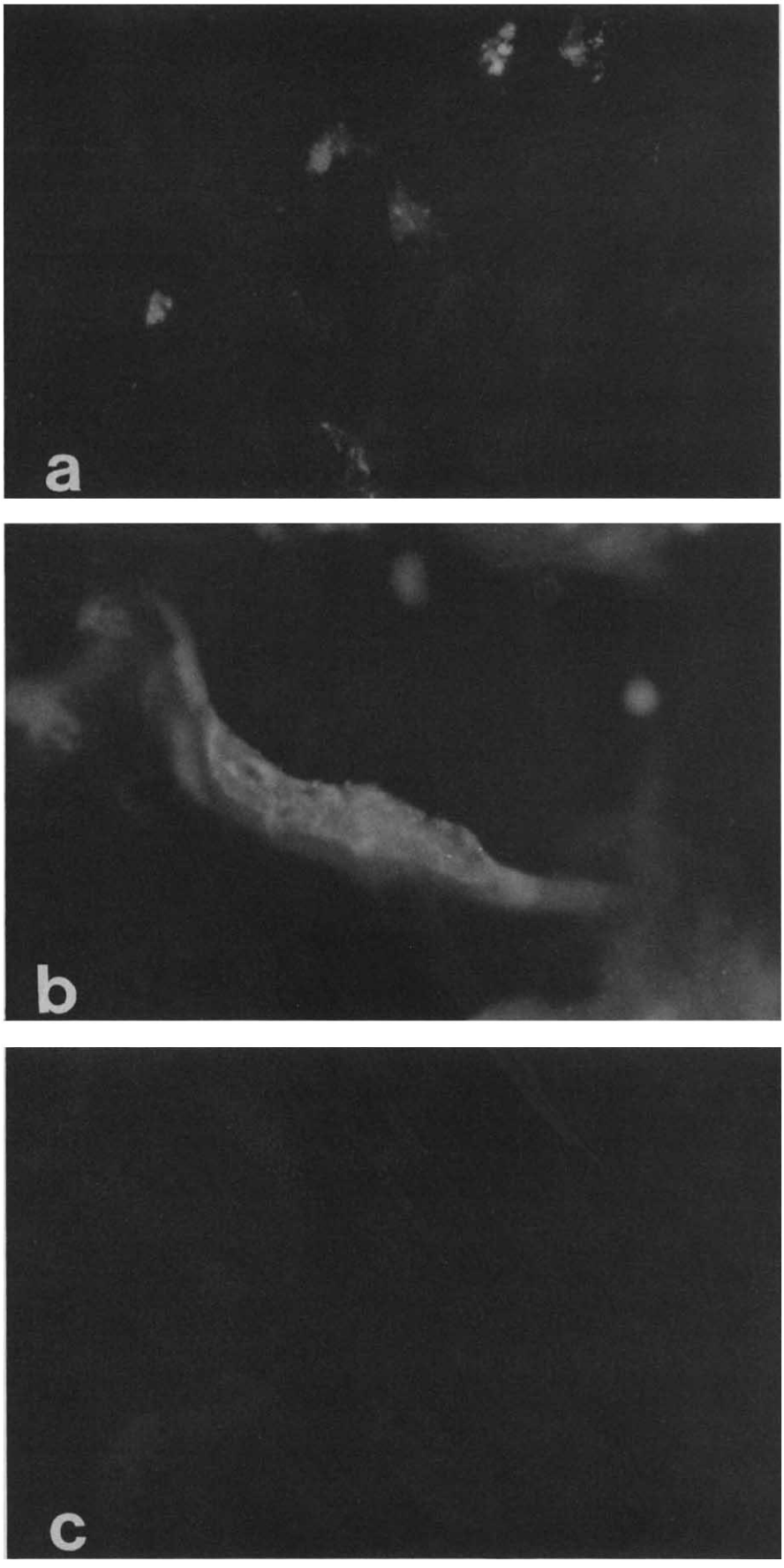

Fig. 8. *Mesangial cells in three-dimensional culture synthesize the basement membrane components collagen type IV and V.* Day 1 (**a** and **c**) and day 4 (**b** and **d**) three-dimensional cultures were stained with antibodies to collagen type IV (a and b) and type V (c and d). Original magnification 372×. **Fig. 8. d.**↗

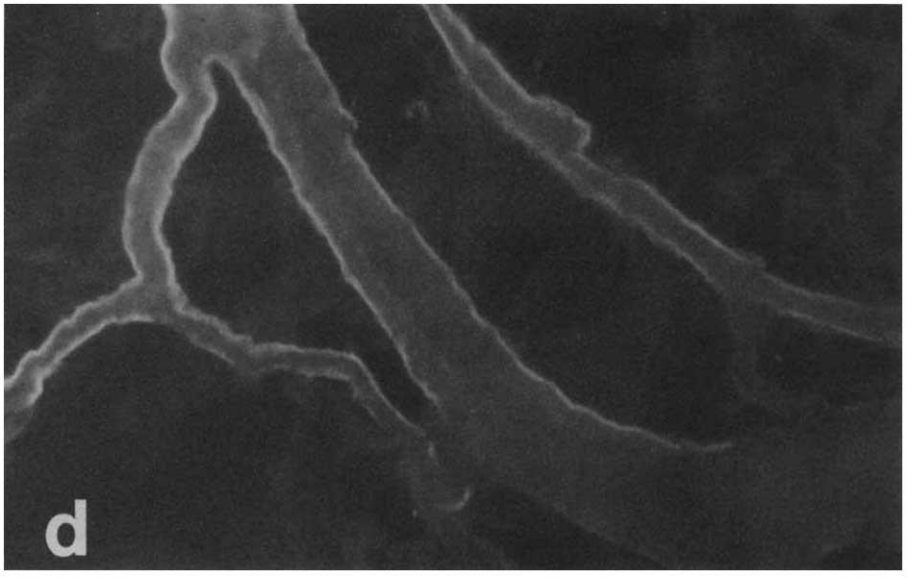

Fig. 8. d.

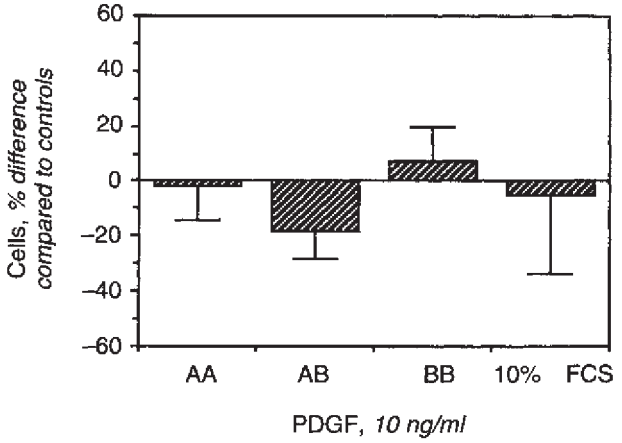

Fig. 9. *Three-dimensional culture prevents the mitogenic effects of PDGF AB and BB.* Mesangial cells were dispersed in type I collagen gels (10^6 cells/ml collagen), made quiescent in serum-free media for 24 hours and treated with PDGF AA, AB and BB (10 ng/ml) or regular growth media supplemented with 10% FCS. After 5 days of culture the gels were digested with collagenase and the cell number determined using an automated cell counter. The data are expressed as relative changes (in %) compared to control cultures in serum free media and given as means ± SD of quadruplicate dishes.

has shown that the composition and spatial organization of ECM profoundly modify the proliferation, migration, biosynthetic activity, cytoskeletal organization, phenotype and differentiation of endothelial and smooth muscle cells [34, 35, 56–59]. In addition, ECM components play an important role in kidney development [60–62] and PDGF has also been recently shown to be a regulator of cell differentiation [63–67]. Therefore we decided to study the impact of different ECM components in conventional two-dimensional culture and of the distinct spatial organization of ECM in three-dimensional culture on the expression of the PDGF receptor subunits and on the mitogenic effects of PDGF in mesangial cells. In 2D culture the α-receptor was only barely detectable by immunoprecipitation from surface labeled cells and the degree of expression was not influenced by ECM. The surface expression and the total amount of the cellular β-receptor protein remained stable even after prolonged culture over seven days on the different ECM components. Consequently, PDGF treatment of mesangial cells in 2D culture on different matrices induced similar mitogenic effects.

On the other hand, the different spatial organization of ECM in 3D culture had a dramatic effect on mesangial cell behavior. A rapid contraction of the 3D collagen gels was observed and indicated a high degree of metabolic activity. Light microscopy revealed profound changes in the mesangial cell phenotype and inter-cellular organization, forming a branching network of multicellular structures. Investigation of the production and deposition of ECM components revealed the induction of the synthesis of collagen type IV and V. This result supports the notion, that the collagen type I gel has just the function of a three-dimensional biological lattice and that the cells rapidly produce and are surrounded by their own extracellular matrix. In addition, the induction of synthesis of the physiological basement membrane components type IV and type V collagen supports the hypothesis that mesangial cells in 3D culture exhibit a differentiated phenotype, closely related to the *in vivo* state. Ultrastructural analysis showed aggregates of activated cells with prominent endoplasmatic reticulum, vacuoles, interdigitization of cell processes and occasionally junctional complexes. In contrast to the high metabolic activity, the cells were in a virtually quiescent proliferative state, comparable to the previously reported low degree of proliferation in 3D cultures [68, 69]. Stimulation with high concentrations of PDGF AA, AB and BB did not induce a mitogenic response. These results prompted us to study the expression of the PDGF-receptors in 3D culture: Immunoprecipitation and immunoblotting studies showed that both PDGF receptor subunits were not expressed on the cell surface in 3D culture and that the total cellular amount of the β-receptor protein decreased rapidly during 3D

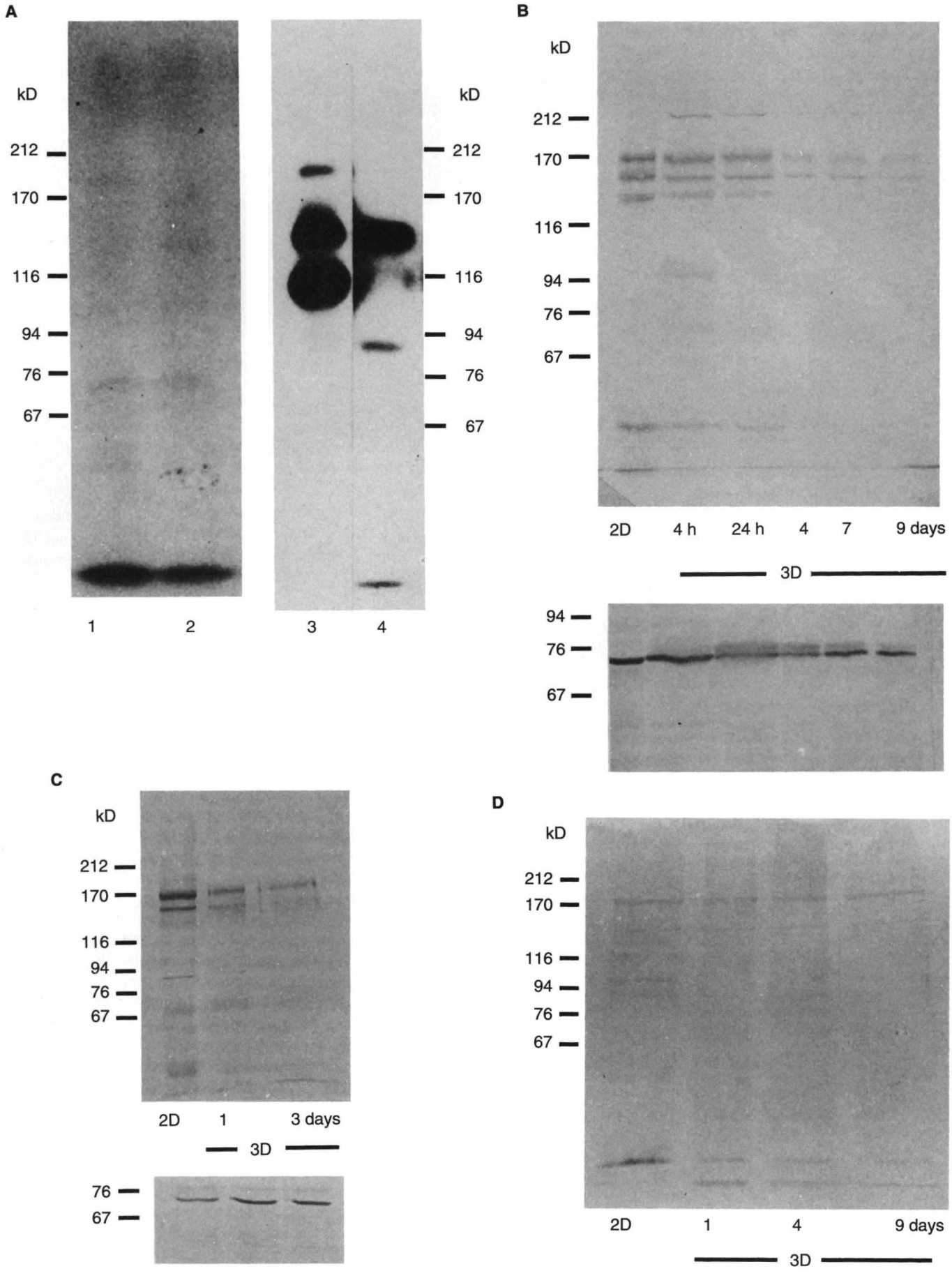
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Fig. 10. *Three-dimensional culture down-regulates the PDGF β-receptor.* Mesangial cells were cultured in type I collagen gels and the PDGF β- and α-receptor subunits were analyzed by immunoprecipitation from ^{125}I labeled cell (**a**). The immunoprecipitation of β_1 and β_3 integrins from the identical cell lysates served as positive control. No surface expression of both PDGF-receptor subunits could be detected (note the high background due to overexposure) after 9 days of three-dimensional culture (Figure 10a, lane 1: β-receptor; lane 2: α-receptor). The strong expression of β_1 (115 kD) and β_3 (80 kD) integrins (a, lanes 3 and 4) immunoprecipitated from the same lysates after 9 days of culture demonstrates the effectiveness of the iodination procedure in the three-dimensional system. The time course of the β-receptor protein (Fig. 7b) was investigated by immunoblotting using lysates prepared at different time points (4 hrs, 24 hrs, 4, 7 and 9 days) during three-dimensional culture, and compared to cells grown in 2D culture. Equal protein loads were analyzed on each lane. **b** shows that not only the surface expression, but the total β-receptor protein also decreased during the time course of three-dimensional culture. The expression of the 70 kD isoform of the cytoskeletal protein 4.1 analyzed from the same cell lysates as the PDGF β-receptor served as control and did not change over the time course of 3D culture (b, lower panel). **c** demonstrates the rapid down-regulation of the PDGF β-receptors during the time course (day 1 and 3) of 3D cultures composed of laminin (0.5 mg/ml) and type collagen (3.5 mg/ml). Protein 4.1 (c, lower panel) was immunoblotted as control. The expression of the PDGF α-receptor (**d**) remained unchanged during the time course (days 1, 4 and 9) of 3D cultures in type I collagen compared to the α-receptor expression in 2D culture.

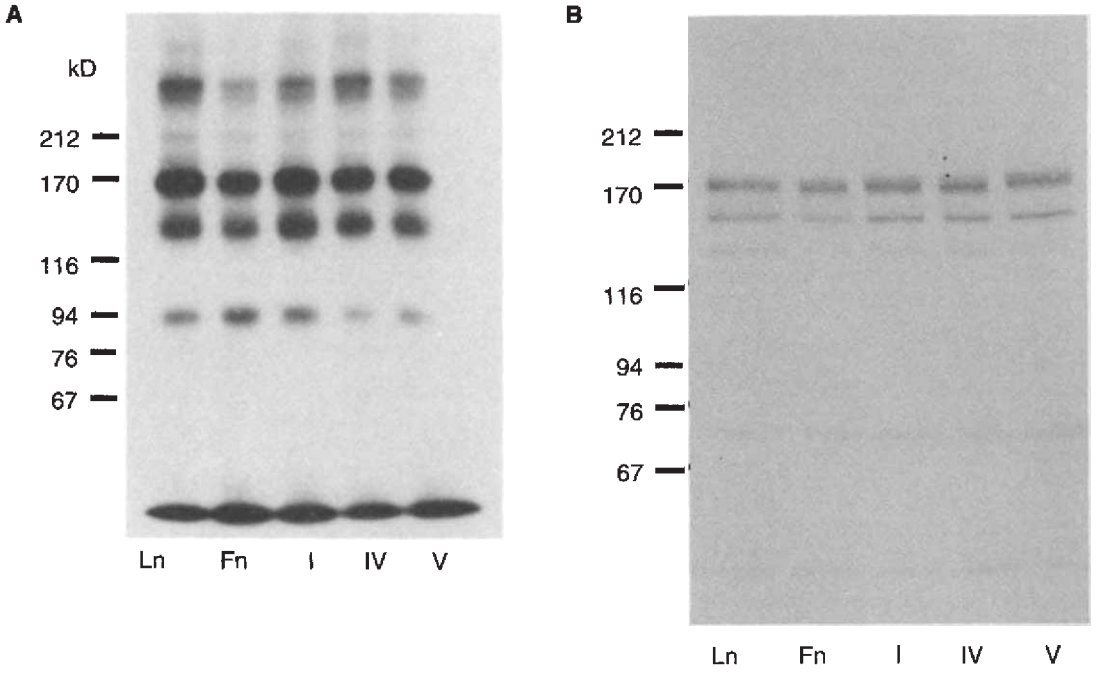

Fig. 11. *Extracellular matrix does not modulate the PDGF β-receptor in 2D culture.* Mesangial cells were plated on laminin (Ln), fibronectin (Fn), type I, IV, and V collagen. Analysis of the β-receptor surface expression by immunoprecipitation from cells labeled with ^{125}I after 7 days of culture revealed no modulation of the β-receptor expression (**a**). In addition, no differences in the total cellular β-receptor protein could be detected by immunoblotting (**b**).

culture. On the other hand, the total cellular amount of the α-receptor, which probably does not transduce mitogenic signals in mesangial cells, remained unchanged. We were preoccupied with the fact, that 3D culture in interstitial type I collagen may be considered a non-physiological environment for mesangial cells, despite of the evidence that the cells rapidly produce their own matrix. Thus we performed several experiments in a matrix to which the physiological basement membrane component laminin was added. The results confirmed our hypothesis considering the spatial organization and not necessarily the composition of extracellular matrix an important modulator of the mesangial cell phenotype. In the modified 3D culture supplemented with laminin the cells remained quiescent and the PDGF β-receptors were rapidly down-regulated. The low mitogenic activity, the down-regulation of the PDGF β-receptor and the failure of PDGF to stimulate cell proliferation in 3D culture cannot be attributed to a decreased cell viability in this particular culture system: the gel contraction, matrix synthesis and cellular organization within the gels are signs of high metabolic activity; the ultrastructural analysis showed highly active, viable cells; and cells isolated from the gels by collagenase digestion were >90% viable by Trypan blue exclusion and attached, spread and proliferated rapidly after replating in conventional 2D culture.

In conclusion, our data show that a differential effect of PDGF isoforms on mesangial cells in 2D culture is due to a particular pattern of PDGF receptor subunit expression and association. In addition, we have shown evidence for a role of the α-receptor subunit in an autocrine regulatory PDGF loop. Furthermore, the different spatial organization in 3D culture dramatically changed the phenotype and mitogenic activity of mesangial cells. A rapid down-regulation of the PDGF β-receptor caused unresponsiveness to exogenous PDGF. Previously, several groups have adduced evidence supporting the notion

that culture of cells normally present in a three-dimensional environment in three-dimensional collagen gels elicits a differentiated cell phenotype and more closely mimics the *in vivo* state. Namely, hepatocytes grown on floating collagen gels assume a trabecular, multicellular organization [70]; mammary epithelial cells assume an acinar organization and secrete increased amounts of milk proteins [71]; MDCK cells form polarized cyst-like structures when grown in type I collagen gels [72] and can be induced to form branching tubule like structures in gels when co-cultured with fibroblasts or with fibroblast conditioned media [73]; and microvascular endothelial cells form differentiated capillary-like multicellular structures when grown in type I collagen gels [35, 46]. Similarly, the mitotically quiescent but metabolically active mesangial cells in 3D culture appear to be more closely related to the *in vivo* behavior of mesangial cells than conventional 2D culture. Our data also demonstrate important roles for the organization of extracellular matrix in the regulation of mesangial cell growth and differentiation. Future studies are focused on the investigation of mechanisms which may induce surface expression of PDGF receptors and PDGF responsiveness in the normally quiescent mesangial cells. These experiments include the analysis of mesangial cells behavior in 3D systems using mixtures of different extracellular matrix components and the evaluation of pro-inflammatory growth factors other than PDGF using this particular culture system.

Acknowledgments

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